Physiological and pathological implications of cholesterol

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1. ABSTRACT

Cholesterol has evolved to fulfill sophisticated biophysical, cell signaling and endocrine requirements of animal systems. At a cellular level, cholesterol is found in membranes, where it increases both bilayer stiffness and impermeability to water and ions. Furthermore, cholesterol is integrated into specialized lipid-protein membrane microdomains with critical topographical and signaling functions. At an organismal level, cholesterol is the precursor for all steroid hormones, including gluco- and mineralo-corticoids, sex hormones and vitamin D, all of which regulate carbohydrate, sodium, reproductive and bone homeostasis, respectively. This sterol is also the precursor for bile acids, which are important for intestinal absorption of dietary lipids as well as energy and glucose metabolic regulation. Importantly, complex mechanisms maintain cholesterol within physiological ranges and the disregulation of these mechanisms results in embryonic or adult diseases, caused by either excessive or reduced tissue cholesterol levels. The causative role of cholesterol in these diseases has been demonstrated by diverse genetic and pharmacologic animal models that are commented in this review.

2. INTRODUCTION

Cholesterol was first identified in 1769 in human gallstones (1). Since then, it has been connected to numerous human diseases such as atherosclerosis, Alzheimer's disease and diverse malformative syndromes. This lipid molecule is essential for membrane structure and function, cell signaling, morphogenesis, intestinal lipid digestion and absorption, reproduction, stress response, sodium and water balance, and calcium and phosphorous metabolism. Research on cholesterol has played important roles in the progress of diverse biomedical fields with thirteen scientists having been awarded with the Nobel Prize for their studies on different aspects of this molecule (2, 3).

Cholesterol is a 27-carbon polycyclic lipid molecule organized in four fused rings in a planar conformation. It has one unsaturated double bond (C5-C6), one β-hydroxyl substitution (C3) and a simple 8-carbon aliphatic tail (Figure 1A). In contrast, plant sterols, called phytosterols, have bulky side chains substituted by methyl and/or ethyl groups (Figure 1B-C). Notably, despite their structural similarities, animals have evolved very efficient
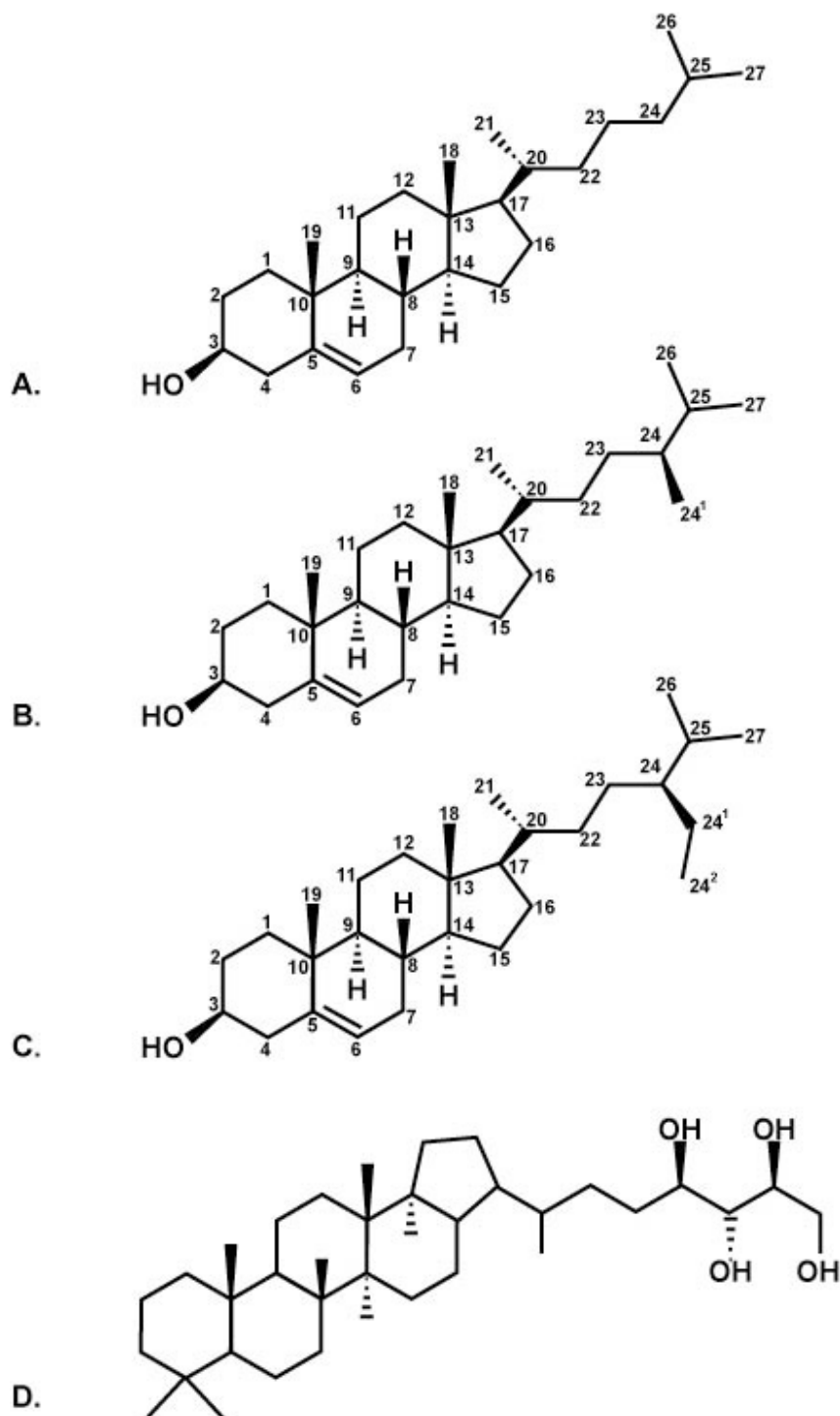
Figure 1. Cholesterol, phytosterols and hopanoids structure. Cholesterol (A) is a 27-carbon, planar, 3β-hydroxylated sterol molecule. This structural features are essential, since minor modifications preclude cholesterol biological functioning. For example, epicholesterol, which has a 3α-hydoxyl group instead of a 3β-hydroxyl group, cannot replace cholesterol in membranes. Campesterol (B) and β-sitosterol (C) are the two more abundant phytosterols in human diet (95% of total plant sterols in a regular diet). They are structurally related to cholesterol, but bulky substitutions in the side chain make them more rigid and hydrophobic, enabling them to prevent proton leaks in plant cell membranes. Bacteriohopane-32,33,34,35-tetrol (D) is the most abundant hopanoid in nature. Hopanoids are anaerobically derived from squalene and, like sterols, are multi-ring molecules that are embedded in membrane monolayers. In there, hopanoids possible play important roles in limiting proton membrane permeability. Sterols carbon numbering system is shown (A-C).

mechanisms to prevent phytosterol accumulation, suggesting that plant sterols cannot fulfill cholesterol functions and/or exert toxic actions on animal systems. The large number of genes involved in cholesterol synthesis, catabolism, trafficking and excretion further support the hypothesis that this sterol is critical for animals.

Importantly, cholesterol cannot be used as an energy source since no enzymes exist that can break the sterol core down to its original acetyl-CoA units.

3. CHOLESTEROL AND MEMBRANE STRUCTURE AND PHYSIOLOGY

Cholesterol is virtually insoluble in water and therefore, it is exclusively found in membranes and other lipid and/or lipid-protein complexes, such as lipid droplets and lipoproteins. Within membranes, cholesterol interacts with phospholipids and sphingolipid fatty acyl chains while simultaneously increasing membrane bilayer rigidity and reducing water and ion membrane permeability (4).

At a subcellular level, cholesterol is heterogeneously distributed, with only 0.5-1% of total cell cholesterol present in the endoplasmic reticulum (ER) (5) and 60-80% in the plasma membrane (6, 7). In this latter organelle, cholesterol accounts for 20% of total lipid mass (8) and appears to be critical for its organization and function. In fact, membrane microdomains called "lipid rafts", which are dynamic protein/lipid assemblies that drift in the liquid-disordered membrane bilayer and are important for extracellular ligand binding as well as for intracellular signal transduction (9), can only be formed when membrane cholesterol concentration reaches a 10% threshold content (10).

Paradoxically, in spite of its importance in animal cells, cholesterol can only be synthesized by vertebrates. Arthropods and nematodes lack critical cholesterol biosynthetic enzymes, squalene synthase and lanosterol synthase as shown below, and must obtain cholesterol from dietary sources (11, 12)

Interestingly, and perhaps reflecting marked inter-species disparities in cholesterol availability, cholesterol requirements are also dramatically different among species. Biochemical determinations have shown that *Caenorhabditis elegans* has 20-fold lower total cholesterol content (normalized to phosphatidylcholine) than mammalian cells. More strikingly, filipin staining studies have shown that these nematodes exhibit groups of completely cholesterol-free cells (13, 14). Intriguingly, despite these very low requirements, cholesterol is still essential for worms, since a synthetic cholesterol enantiomer, which has identical biophysical properties, cannot support the growth and reproduction of these worms (15).

Prokaryotic organisms, on the other side, have evolved alternative molecular strategies to carry out the membrane-organizing properties of sterols. In fact, either complex linear carotenoids or squalene-derived cyclic hopanoids (Figure 1D) are found across bacterial species,

increasing membrane rigidity and reducing membrane permeability to water and cations. Interestingly, since both squalene cyclization to form lanosterol and lanosterol demethylation to generate cholesterol require molecular oxygen (Figure 2), it has been speculated that prokaryotes evolved hopanoids before the appearance of molecular oxygen in the atmosphere, resulting in a convergent adaptation of cell membranes to increasingly complex environments (8).

4. CHOLESTEROL BIOSYNTHESIS

The elucidation of cholesterol's structure and synthesis was determined through the works of Nobel laureates H.O Wieland (1927), L. Ruzicka (1939), R. Robinson (1947), K. Bloch and F. Lynen (1964) and J. W. Cornforth (1975) (16, 17).

Cholesterol is synthesized in the endoplasmic reticulum (ER) by the action of over 30 enzymes organized in the mevalonate pathway (Figure 2) whose first part involves four fundamental steps: 1) condensation of three acetyl-CoA units to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA); 2) HMG-CoA reduction mediated by NADPH to generate mevalonate (a 30-carbon linear molecule); 3) conversion of mevalonate into activated isoprenoids 3-isopentenyl pyrophosphate and dimethylallyl pyrophosphate; and 4) polymerization of six isoprenoids units into squalene. Next, linear squalene undergoes series of oxygenation and cyclization to form lanosterol, and finally, lanosterol is converted to cholesterol by sequential oxidative demethylations and double bond isomerizations and reductions.

The rate-limiting reaction in cholesterol biosynthesis is the conversion of HMG-CoA into mevalonate catalyzed by HMG-CoA reductase (HMG-CoA-R). This enzyme is tightly regulated at both transcriptional and post-translational levels and is the pharmacological target of statins, the most potent cholesterol lowering agents currently available.

Importantly, isoprene intermediates in the mevalonate pathway generate a variety of other bioactive molecules, including ubiquinone and heme A, involved in the mitochondrial electron transport; dolichols, involved in glycoprotein synthesis; isopentyladenine, present in some transfer RNAs; and farnesyl and geranyl groups required for membrane proteins prenylation (17).

5. CELLULAR CHOLESTEROL UPTAKE

In addition to being endogenously synthesized, cholesterol is incorporated into the cell from plasma lipoproteins by cell surface receptor mediated endocytosis (Figure 3). Brown and Goldstein first dissected the now prototypical low density lipoprotein (LDL) receptor (LDLR) pathway (18), demonstrating that specific cell surface receptors are required for extracellular ligand endocytosis (2). In this model, circulating LDL binds to LDLR on the cell surface and are incorporated, as a whole lipoprotein-receptor complex, via clathrin-coated vesicles,
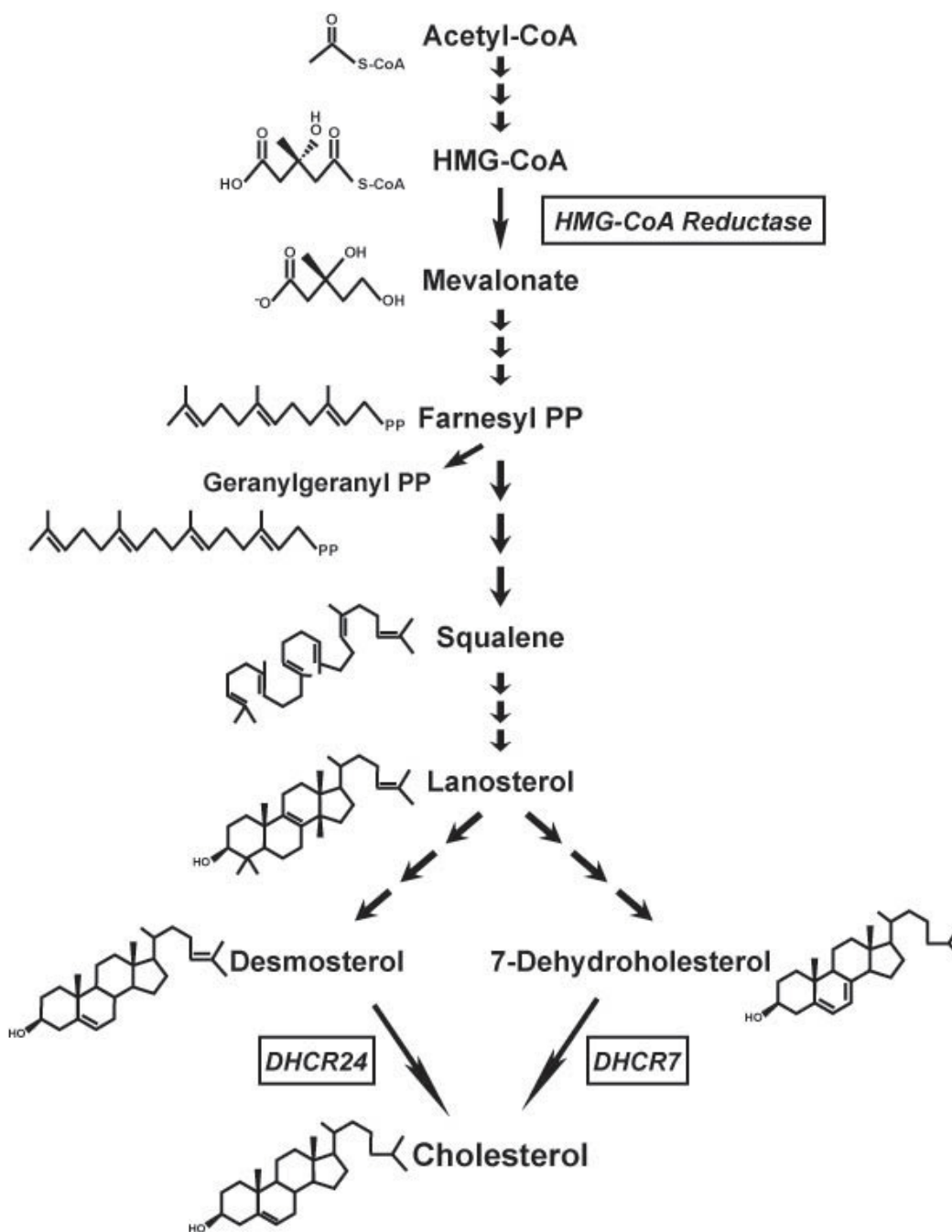
Figure 2. Cholesterol synthesis in the mevalonate pathway. First, 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) and mevalonate are formed from acetyl-CoA moieties. The conversion of HMG-CoA into mevalonate is catalyzed by HMG-CoA reductase. This is the only limiting reaction in cholesterol synthesis and is the target of statins cholesterol lowering drugs. Mevalonate is converted into activated isoprenoids isopentenyl pyrophosphate and methyallyl pyrophosphate (not shown), which, in turn, originate farnesyl pyrophosphate and squalene. The subsequent cyclization and oxygenation of squalene to lanosterol involves the action of squalene monooxygenase and lanosterol synthase (also known as oxidosqualene cyclase, not shown) and requires molecular oxygen and NADPH. These reactions only happen in vertebrates. Finally, the generation of cholesterol involves lanosterol: 1) demethylation at C4α, C4β, and C14; 2) isomerization of the $\Delta^{8(9)}$ double bond to a Δ^7 double bond; 3) desaturation to form a Δ^5 double bond; and 4) reduction of Δ^{14}, Δ^{24}, and Δ^7 double bonds. Importantly, the reduction of Δ^{24} double bond, catalyzed by 3β-hydroxysterol Δ24-reductase (DHCR24) can happens at any level below lanosterol, originating two parallel pathways and resulting in either 7-dehydrocholesterol or desmosterol, which are reduced to cholesterol by 7-dehydrocholesterol reductase (DHCR7) or DHCR24, respectively.

into endolysosomal compartments for further processing. At this level, LDLR is recycled back to the surface, whereas LDL particles are fully degraded into their individual components. More specifically, LDL-derived cholesteryl esters are de-esterified to form free cholesterol and fatty acids by the action of lysosomal acid lipase. Deficiency of this enzyme leads to the buildup of cholesteryl esters and other lipids in tissues, causing a lysosomal storage disorder known as Wolman's disease.

Niemann Pick type-C disease, a lethal condition characterized by intracellular unesterified cholesterol accumulation, is caused by mutations in *Npc1* and *Npc2* genes that preclude endolysosomal cholesterol export (19). NPC1 is a large multispan lysosomal integral membrane protein that binds cholesterol at the 3β hydroxyl position (20). NPC2, by contrast, is a soluble protein located within the lysosomal lumen that binds cholesterol on its aliphatic side chain (21). Brown and Goldstein´s group recently demonstrated that lysosomal cholesterol export requires the concerted action of both NPC proteins, possibly involving the transfer of cholesterol from NPC2 to NPC1 and the subsequent insertion of cholesterol's aliphatic side chain into the lysosomal membrane. Endolysosomal cholesterol is then exported to the ER and incorporated in the metabolically active intracellular pool, by mechanisms that have yet to be elucidated.

In contrast to LDL cholesterol uptake, high density lipoprotein (HDL) cholesterol is incorporated into cells by non-endocytic mechanisms (Figure 3). The scavenger receptor class B type I (SR-BI), an integral membrane protein found mostly in the liver and steroidogenic tissues, directly binds HDL particles and facilitates the selective transfer of HDL cholesterol into the plasma membrane with no internalization of the holoparticle (22). Cholesterol-depleted HDL particles are released back into the extracellular space and the transferred cholesterol is hydrolyzed by extralysosomal neutral cholesteryl ester hydrolases and trafficked towards the ER by unknown mechanisms (23).

6. CELLULAR CHOLESTEROL EFFLUX

Several ATP-binding cassette (ABC) transporter superfamily members efflux cholesterol towards extracellular acceptors (Figure 3). ABC subfamily A, member 1 (ABCA1) transfers plasma membrane cholesterol into lipid-poor discoidal HDL precursors. Tangier's disease, a condition caused by ABCA1 deficiency, is characterized by very low plasma HDL levels and cholesterol accumulation in macrophages, possibly as a result of defective cholesterol exportation (24). ABCG1, which is also expressed in macrophages, effluxes cholesterol towards already lipid-loaded spherical HDL particles (25), possibly contributing to the full maturation of this lipoprotein class *in vivo*. SR-BI has also been reported to play a role in cholesterol efflux into mature HDL particles (22).

ABCG5 and ABCG8 transporters are expressed on the canalicular surface of hepatocytes as well as on the luminal membrane of enterocytes. In this location, both transporters actively pump cholesterol and phytosterols out of cells, contributing to the net elimination of these sterols from the body (26). Concordantly, sitosterolemia, a genetic disease caused by mutations in *ABCG5* and *ABCG8* genes, is characterized by phytosterol and cholesterol accumulation, high plasma sterol concentration and accelerated atherosclerosis, indicating that these proteins function to eliminate sterols in humans (27).

Cholesterol in the bile has classically been thought to correspond to a mere manifestation of body cholesterol deposition. However, experimental evidence suggests that bile cholesterol, associated with phospholipids in lipid lamellae, may have a physiological role, protecting both biliary tree and intestinal epithelium against the toxic actions of bile salts *in vivo* (28).
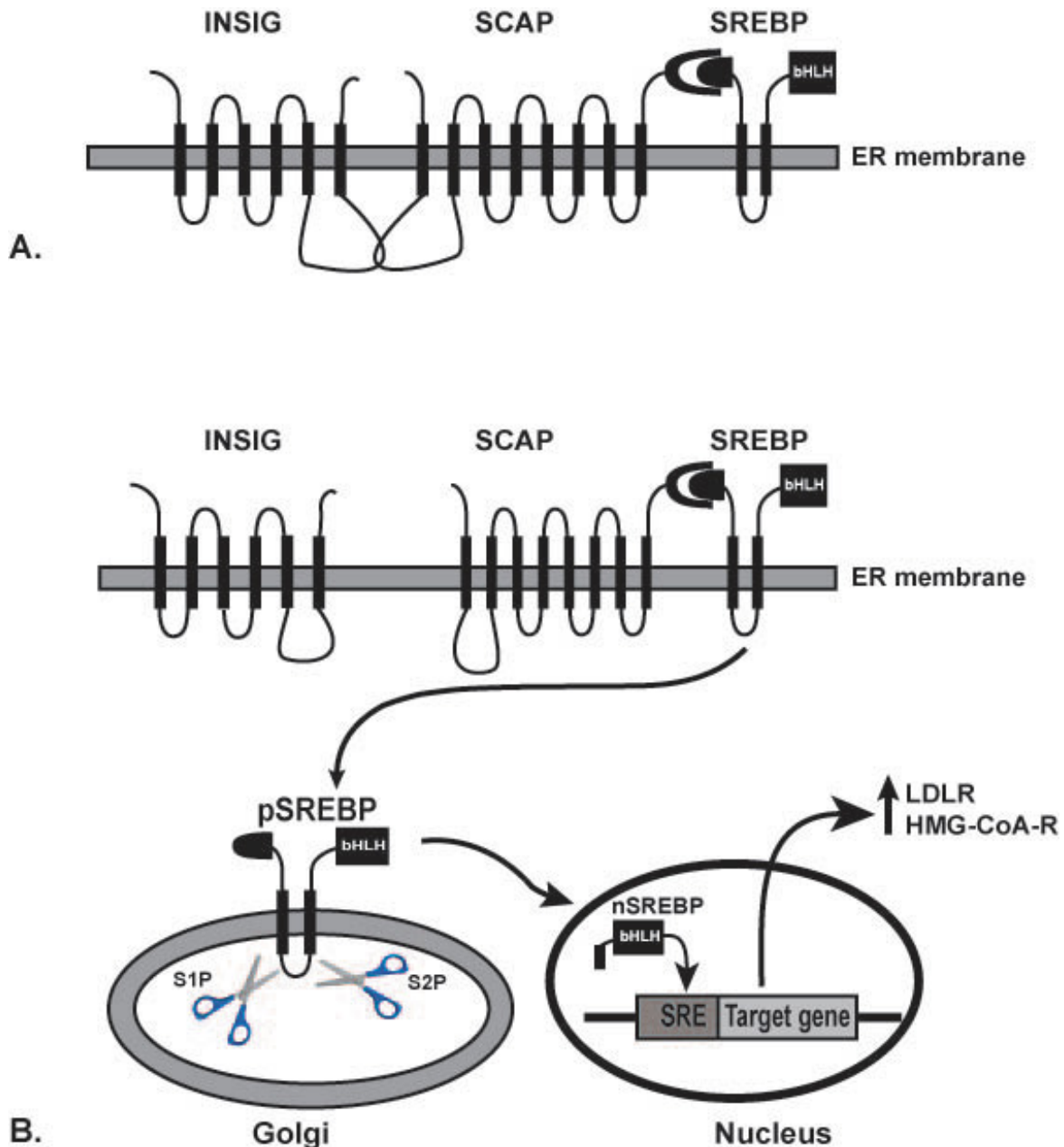
7. INTRACELLULAR CHOLESTEROL REGULATION

Cholesterol may be deleterious or even lethal for cells and many protective mechanisms have evolved to prevent this cellular toxicity. The underlying causes of cholesterol's cytotoxicity are not completely clear, but may include: 1) abnormalities in membrane proteins conformational plasticity, 2) increased production of oxysterols by oxidative stress, 3) intracellular cholesterol crystallization leading to mechanical membrane disruption, and 4) increased apoptosis (29).

Cellular cholesterol is kept within homeostatic levels by the concerted action of both transcriptional and post-transcriptional mechanisms (30, 31). At the transcriptional level, sterol regulatory element binding protein (SREBP), a membrane bound member of the basic helix-loop-helix family of transcription factors, controls the gene expression of LDLR, HMG-CoA reductase and other genes encoding enzymes of the mevalonate pathway (Figure 4). SREBP-cleavage activating protein (SCAP), a multi-spanning ER membrane protein, directly binds cholesterol and interacts with SREBP and other ER proteins to regulate SREBP activation. Specifically, when ER cholesterol levels are high (Figure 4A), SCAP physically interacts with insulin-induced genes 1 and 2 (INSIG1 and INSIG2) (32, 33) and the SCAP/SREBP complex is retained in the ER preventing its translocation towards the golgi. In contrast, when cholesterol levels are low (Figure 4B), the SCAP-INSIGs interaction is abolished, and the SCAP/SREBP complex is transported into the golgi, where SREBP is released from the membrane by the sequential action of site-1 and site-2 proteases (S1P and S2P, respectively). This mature membrane-free SREBP is translocated into the nucleus where it binds to sterol responsive element (SRE) sequences of target genes, activating their transcription.

HMG-CoA reductase is additionally regulated at the post-translational level. When ER cholesterol concentration is high, HMG-CoA reductase directly binds to cholesterol and to a membrane ubiquitin ligase, gp78. This interaction results in HMG-CoA reductase
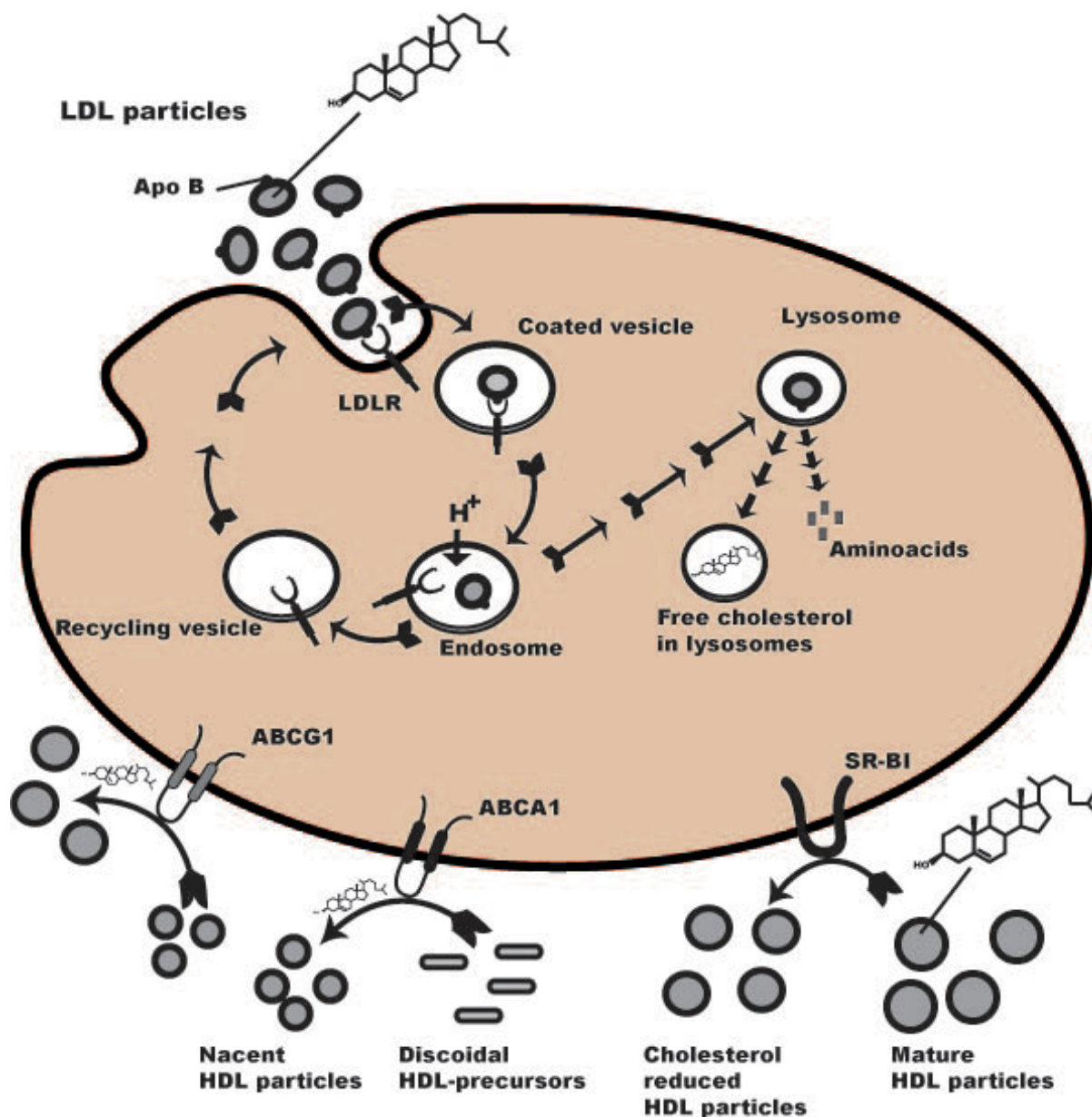
Figure 3. Cholesterol uptake and efflux are mediated by cell surface proteins. Low-density lipoprotein (LDL) binds to LDL receptor (LDLR) in clathrin-coated pits on the surface of numerous cell types, including hepatocytes, corticoadrenal epithelium, macrophages and skin fibroblasts. After binding, the LDL-LDLR complex is internalized and transferred to endosomes, where, by acidification of the organelle, it dissociates into free LDL and LDLR. The LDLR is recycled back to the surface, for new binding/internalization rounds. The LDL particles, by contrast, are trafficked to lysosomes for full degradation by the action of lysosomal proteases and lipases. The released unesterified cholesterol is transported to the plasma membrane and the endoplasmic reticulum for structural or metabolic roles, or is re-esterified by acyl-coA:cholesterol acyltransferase (ACAT, not shown) for storage in lipid droplets. High-density lipoprotein (HDL) binds to a different cell surface protein, the Scavenger Receptor class B member I (SR-BI) and is not internalized after its binding. In fact, cholesterol and cholesteryl-esters are selectively transferred from HDLs to intracellular regulatory pools, with no endocytosis or degradation of HDL particles. As a result, mature HDLs are converted into smaller, cholesterol-reduced but still-spherical lipoproteins. Efflux of cellular cholesterol is mediated by ATP binding cassette (ABC) family members. ABCA1, a multiple membrane-spanning protein with two nucleotide-binding domains, interacts with discoid lipid-poor apo A-I containing HDL precursors, transferring phospholipids and cholesterol to it. These lipidated apo A-I containing precursors are then transformed into HDL, in the blood, by the action of Lecithin:cholesterol acyl transferase (LCAT, not shown). Finally, ABCG1 incorporates additional cholesterol to HDL particles, allowing their full maturation.

Figure 4. Cellular cholesterol is regulated at transcriptional level by SREBP system. SCAP, a multi-span ER membrane protein, binds cholesterol through its luminal loop 1. This binding stabilizes SCAP interaction with another ER resident protein, named INSIG (A), anchoring the SCAP-SREBP complex in the ER and preventing SREBP activation. When ER cholesterol is "low" (B), cholesterol detaches from SCAP and releases the INSIG-SCAP interaction, as a consequence of SCAP conformational changes. Simultaneously, this action exposes COPII binding sites in the cytosolic loop 6 of SCAP, enabling the migration of SCAP-SREBP complex to the Golgi. In there, the precursor (p)SREBP is sequentially processed by site 1 protease (S1P) and site 2 protease (S2P), which releases a mature membrane-free SREBP. This basic-helix-loop-helix (bHLH) containing fragment translocates to the nucleus and binds to sterol responsive elements (SREs) in the regulatory region of SREBP target genes, such as LDLR, HMG-CoA-R among others, elevating their transcription rates.

polyubiquitination and subsequent 26S proteasome-mediated degradation (30, 34). Conversely, when ER cholesterol is low, HMG-CoA reductase protein turnover rate is reduced as a result of decreased polyubiquitination and proteosomal degradation.

Statins, the main hypocholesterolemic drug, work by indirectly activating the SREBP system. Statins competitively inhibit HMG-CoA reductase activity, which results in reduced ER cholesterol levels. This change increases SCAP-modulated SREBP activation and,

consequently, elevates LDLR levels on cell surface. This, in turn, accelerates LDL clearance rates and reduces plasma LDL cholesterol concentrations.

Importantly, members of the nuclear receptor superfamily such as liver X receptor (LXR) and farnesoid X receptor (FXR) have recently been identified as additional transcriptional regulators of the synthesis, transport and catabolism of cholesterol in different tissues (35).

8. CHOLESTEROL IN EMBRYONIC AND FETAL DEVELOPMENT

Several human malformation syndromes as well as animal models of pharmacologic or genetic blockade of the mevalonate pathway strongly suggest that cholesterol is required for normal prenatal development (36). These syndromes most likely result from structural, endocrine and/or signaling abnormalities derived from cholesterol deficiency (37, 38). However, it is plausible that reduction and/or accumulation of other mevalonate pathway metabolites play a role as well.

Because of their high proliferation and membrane formation rates, embryonic and fetal cells have elevated cholesterol requirements. Interestingly, these cells do not suppress cholesterol synthesis in response to elevated ER sterols concentration (39, 40), possibly as an adaptation to secure cholesterol supply. Mechanistically, this lack of feedback regulation seems to result from SREBP constitutive processing and activation, as a consequence of elevated SCAP/INSIG ratios in the ER membrane (39).

The most prevalent genetic disease involving reduced embryonic cholesterol is the Smith-Lemli-Opitz (SLO) syndrome, which is caused by a defective conversion of 7-dehydrocholesterol (7-DHC) into cholesterol and is characterized by facial dysmorphia, microcephaly, syndactyly and variable degrees of mental retardation. Importantly, both mice lacking 3β-hydroxysteroid Δ7-reductase ($Dhcr7^{-/-}$ mice) and rat embryos treated with the DHCR7 inhibitor AY9944, recapitulate human SLO (41), indicating that cholesterol restriction and/or 7-DHC accumulation cause this disease.

In *Drosophila,* which cannot synthesize cholesterol (see above), activities of HMG-CoA reductase, geranylgeranyl pyrophosphate synthase and geranylgeranyl transferase are required for normal heart development (42). This indicates that other metabolites of the mevalonate pathway rather than cholesterol, are required for development. Supporting this idea, it has been shown that statins induce an abnormal cardiac phenotype in flies, possibly due to the mislocalization of unprenylated G proteins and disruption of normal morphogenic cell signaling. (42). Remarkably, heart malformations have also been reported after gestational exposure to statins in humans (43), suggesting that a normal metabolic flux through the mevalonate pathway is required in human embryonic development as well.

Paradoxically, excessive tissue cholesterol seems to be as embryotoxic as reductions of this sterol. In fact, *Insig1/Insig2* double knockout mice, which have elevated SREBP levels and therefore increased mevalonate pathway activity, develop palate or facial clefting because of defective midline fusion (44). In these animals, statin administration to pregnant females largely prevents the malformation phenotype, suggesting that elevated sterols or other mevalonate pathway-related metabolites somehow precludes the normal fusion of midline embryonic structures (44).

9. ORIGINS OF EMBRYONIC/FETAL CHOLESTEROL

Experimental evidence indicates that cholesterol is actively transferred from mother-to-embryos/fetuses during their pregnancy. First, fetuses with defective endogenous cholesterol synthesis still have considerable amounts of this sterol in their tissues (45, 46). Second, labeled LDL and/or HDL cholesteryl esters injected into maternal circulation are quickly found in fetal blood and tissues (47-49). Finally, maternal lipoprotein metabolic abnormalities negatively impact embryonic development. Indeed, low maternal plasma cholesterol correlates with low weight at birth and microcephaly in humans (50), whereas hypercholesterolemia (beyond its physiological gestational increase) associates with fatty streaks in the fetal aorta (51). Additionally, offspring of female mice lacking endocytic HDL receptor megalin (52) or apolipoprotein B (53) develop various cephalic and central nervous system abnormalities (37).

From an anatomical point of view, maternal cholesterol must cross two different cell layers before reaching fetal circulation. These are the visceral endoderm in the yolk sac or trophoblast in the placenta, and fetal endothelial cells (54). Although the precise molecular mechanisms underlying this trafficking remain unknown, both trophoblast and placental endothelial cells express lipoprotein receptors and sterol transporters that likely mediate this transfer *in vivo* (55, 56).

Since cholesterol is critical for normal embryonic development, improving mother-to-fetus cholesterol movement could be envisioned as a potential *in utero* therapy for conditions in which embryonic cholesterol synthesis is restricted, such as SLO syndrome. In fact, increasing ABCA1 and cholesterol transport significantly increases tissue cholesterol content in murine fetuses that lack cholesterol synthesis (57).

10. CHOLESTEROL CYTOTOXICITY: IMPLICATIONS FOR ATHEROSCLEROSIS

Atherosclerotic disease is the major cause of cardiovascular death worldwide, and although it is a complex process in which various environmental and genetic factors are involved, the deposition of lipoprotein-derived cholesterol in the arterial wall is the priming condition necessary for more advanced lesions. Strikingly, Anitschkow recognized the role of cholesterol in

atherogenesis in 1913; unfortunately, such an advance was not considered by contemporary scientific authorities, delaying progress of the field for decades (58).

After the initial cholesterol buildup in the arterial wall, local inflammation and endothelial dysfunction further contribute to additional cholesterol accumulation and, in some cases, atherosclerotic plaque rupture and thrombosis (59, 60). Importantly, acute ischemic atherothrombotic events invariably occur in cholesterol-rich lesions; however, the mechanisms precipitating these plaque complications and its links to cholesterol remain unknown. Cell death of free cholesterol-loaded macrophages, mainly by apoptosis, has been proposed as a major precipitating factor (61) and direct toxic effects of free cholesterol on cellular membranes or activation of death-promoting signaling molecules, or a combination of both, are likely involved in precipitating these complications. Consistently, the inhibition of acyl-coenzyme A: cholesterol acyltransferase (ACAT), an intracellular enzyme involved in cholesterol esterification, leads to macrophage cell death (62-64), suggesting that free cholesterol is directly toxic for lesional macrophages. Conversely, NPC1 mutant mice, which have a defective cholesterol trafficking from lysosomes to the ER and accumulate unesterified cholesterol in endolysosomal compartments (see above), are protected from cholesterol-induced macrophage apoptosis and atherosclerosis (65), possibly because cholesterol in lysosomes is inaccessible to the machinery implicated in cell death.

Recently, activation of ER stress compensatory pathways, known as the unfolded protein response (UPR), has also been implicated in atherosclerosis (66). In particular, high levels of cholesterol, oxysterols and saturated fatty acids directly activate the UPR in plaque macrophages (67), promoting its progression towards more advanced lesions. Chronic UPR activation, therefore, represents an alternative cholesterol pathogenic pathway that opens new translational research fields.

11. IS CHOLESTEROL ESSENTIAL FOR LIFE?

This question, challenging the general wisdom in the field, was raised after the generation of the so-called "cholesterol-free" mice. Desmosterol reductase deficient mice (*Dhcr24*$^{-/-}$), which cannot catalyze the final step of cholesterol biosynthesis (Figure 2), were generated to model the human disease desmosterolosis (68). Surprisingly and in sharp contrast to human patients (69, 70), these mice survived and reached adulthood in spite of virtually undetectable levels of cholesterol in the blood, liver and central nervous system.

So, why is cholesterol essential for some species (humans), but not for others (mice)? The answer may lie in interspecies differences in the maternal availability of cholesterol during embryonic development and/or the ability of desmosterol to fulfill the roles of cholesterol against suboptimal cholesterol concentrations among species. Indeed, although desmosterol can replace cholesterol in membranes, with no negative biophysical

effects (71) and regulate the SREBP pathway and support cell proliferation in *in vitro* systems (72), it is unable to replace cholesterol in whole human organisms, such as desmosterolosis disease shows.

However, it is in the clinics where this question about cholesterol essentiality has immediate relevance. This is because all major clinical strategies to reduce cardiovascular risk are centered around reducing LDL cholesterol. Indeed, the American Heart Association (AHA)/American College of Cardiology Foundation (ACCF) guidelines recommend that in very high-risk patients, such as those that have previously suffered a myocardial infarction, LDL cholesterol should be lowered to less than 70 mg/dl (73), implying the use of potent statins to antagonize the mevalonate pathway.

The point is, therefore, how deeply can plasma cholesterol be lowered before having adverse clinical events? The answer remains unknown. In fact, apparently contradicting evidence makes this task particularly complex. On one hand, patients with non-sense mutations of *PCSK9*, a protease involved in LDLR degradation (74), have a 28% reduction in mean LDL cholesterol (100 mg/dl vs. 140 mg/dl) and a 88% reduction in their cardiovascular risk relative to non-carrier individuals (75) More importantly for this discussion, in this cohort, even the more intensely hypocholesterolemic-*PCSK9* mutant carriers (with LDL cholesterol as low as ~ 40 mg/dl) remained free of any health complications. Supporting the safety of this markedly reduced plasma cholesterol concentration, the mean LDL cholesterol found in the cord blood of 2,937 healthy newborns was ~ 50 mg/dl (76), suggesting that LDL cholesterol could be drastically reduced as a cardiovascular goal with no adverse effects on health.

However, previous experiences of cholesterol synthesis inhibition with triparanol, a DCHR24 inhibitor (Figure 2) raised serious concerns about the safety of massive cholesterol lowering in humans. In fact, this drug potently reduced plasma cholesterol and it was extensively used in the 1960's to treat hypercholesterolemic patients (77). Unfortunately, it was also associated with severe side effects such as cataracts, intestinal lesions and teratogenesis (78). However, it is theoretically possible that, in the setting of DHCR24 antagonism, these complications could be a direct result of desmosterol build-up rather than cholesterol reduction, or alternatively, that they correspond to triparanol's drug-intrinsic toxicity.

12. FUTURE DIRECTIONS

The long history of cholesterol is full of scientific challenges. Starting with the elucidation of the cholesterol structure and its biosynthesis, and followed by the understanding of its cell and whole organism regulation, cholesterol research has led to some of the most significant biomedical advancements of the last century, providing significant therapeutic applications (e.g., statin development) and sparing millions of lives worldwide. However, as summarized here, this knowledge is far from

complete. Further research will be needed to continue delivering basic findings with strong translational implications for human health. It is our hope that the antecedents shown here will inspire people to explore cholesterol biology in their favorite fields.

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